Golgi-to-Late Endosome Trafficking of the Yeast Pheromone Processing Enzyme Ste13p Is Regulated by a Phosphorylation Site in its Cytosolic Domain

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This study addressed whether phosphorylation regulates trafficking of yeast membrane proteins that cycle between the trans-Golgi network (TGN) and endosomal system. The TGN membrane proteins A-ALP, a model protein containing the Ste13p cytosolic domain fused to alkaline phosphatase (ALP), and Kex2p were found to be phosphorylated in vivo. Mutation of the S13 residue on the cytosolic domain of A-ALP to Ala was found to block trafficking to the prevacuolar compartment (PVC), whereas a S13D mutation generated to mimic phosphorylation accelerated trafficking into the PVC. The S13 residue was shown by mass spectrometry to be phosphorylated. The rate of endoplasmic reticulum-to-Golgi transport of newly synthesized A(S13A)-ALP was indistinguishable from wild-type, indicating that the lack of transport of A(S13D)-ALP to the PVC was instead due to differences in PVC/Golgi/endosomal trafficking. The A(S13A)-ALP protein exhibited a TGN-like localization similar to that of wild-type A-ALP. Similarly, the S13A mutation in endogenous Ste13p did not reduce the extent of or longevity of its localization to the TGN as shown by α-factor processing assays. These results indicate that S13 phosphorylation is required for TGN-to-PVC trafficking of A-ALP and imply that phosphorylation of S13 may regulate recognition of A-ALP by vesicular trafficking machinery.

INTRODUCTION

Resident trans-Golgi network (TGN) membrane proteins of the yeast Saccharomyces cerevisiae frequently cycle between the TGN and endosomal system. Ste13p (dipeptidyl aminopeptidase A) and the endopeptidase Kex2p are TGN resident enzymes with single transmembrane spanning domains that process the mating pheromone α-factor in the TGN. These proteins are delivered to a prevacuolar endosomal compartment (PVC) with a half-time of ~60 min as determined using the Ste13p-based reporter protein A-ALP (Bryant and Stevens, 1997; Nothwehr et al., 1999). Once at the PVC, Ste13p and Kex2p are packaged into retrograde vesicles for delivery to the TGN. This transport step is dependent on a peripheral membrane complex called the retromer that may function as a vesicle coat (Seaman et al., 1997, 1998; Nothwehr et al., 1999). Retromer-based sorting of Ste13p/A-ALP involves the association of retromer subunit Vps35p with an aromatic amino acid-based sorting signal (FXFXD) in the cytosolic domain of Ste13p (Nothwehr et al., 2000). Kex2p and Vps10p also contain aromatic PVC retrieval signals. Mutation of these signals, or loss of retromer function, causes rapid default transport of these cargo to the vacuole (Wilcox et al., 1992; Nothwehr et al., 1993; Cereghino et al., 1995; Cooper and Stevens, 1996).

In addition to a cycling itinerary that includes the PVC, TGN residents seem to also visit early endosomal compartments as shown by several recent studies. For example, Kex2p and Ste13p seem to localize to a certain degree with early endosomes. This has been shown by colocalization with early endosomal markers Tlg1p, Chs3p, and Snclp, a secretory v-SNARE that transits through the early endosomal system on its journey from the plasma membrane to the PVC (Ziman et al., 1995; Santos and Snyder, 1997; Holthuis et al., 1998; Lewis et al., 2000). Both Ste13p and Kex2p contain poorly defined cytosolic domain signals that when mutated accelerate trafficking into the PVC (Brickner and Fuller, 1997; Bryant and Stevens, 1997), suggesting that either they function as static retention signals in the TGN or regulate trafficking through the early endosomal system. Mutation of the yeast synaptotagmin Inp53p also caused A-ALP (as a model for Ste13p) and Kex2p to be more rapidly delivered to the PVC; however, trafficking of Vps10p was unaffected (Ha et al., 2001). The phenotypes associated with a loss of Inp53p function and a loss of the AP-1 adaptor complex share similarities in that both types of lesions exhibit synthetic growth defects when combined with mutations in GGA1 and GGA2 (Costaguta et al., 2001; Ha et al., 2003). GGA1 and GGA2 encode adaptor proteins necessary for clathrin-mediated transport from the TGN directly to the PVC (Black and Pelham, 2000; Costaguta et al., 2001; Scott et al., 2004). The synthetic growth defects obtained with mutations in the AP-1 adaptor complex or Inp53p with mutations in the GGAs suggest that AP-1 and Inp53p probably mediate a
distinct pathway into the endosomal system, presumably to early endosomes. This assertion also is supported by the observation that trafficking of Chs3p and Tlg1p between the TGN and early endosomes is disrupted by mutations in AP-1 (Valdivia et al., 2002). Finally, mutation of SO13/RAV1, which is required for efficient transport between the early endosomes and the PVC, reduced the rate of trafficking of Kex2p to the PVC (Sipos et al., 2004). Together, these results suggest that Ste13p and Kex2p cycle between the TGN and early endosome and also reach the PVC via transport from the early endosome.

With yeast TGN resident proteins engaging in such a complex trafficking itinerary, there is clearly more to learn regarding the regulation of trafficking between the different compartments. Phosphorylation of membrane proteins that cycle within the TGN/endosomal system of mammalian cells is known to influence their trafficking. In some cases, phosphorylation of a serine or threonine influences the activity of a nearby sorting signal, whereas in other cases phosphorylation generates a new sorting signal function by binding to an accessory protein (Bonifacio and Traub, 2003; Hinners and Tooze, 2003). For example, TGN localization of the Kex2p homologue furin is dependent on an acidic cluster motif in its cytosolic domain (Schäfer et al., 1995; Takahashi et al., 1995; Voorhees et al., 1995). This motif contains a serine that can be phosphorylated by casein kinase II (Jones et al., 1995). In response to phosphorylation, furin is thought to cycle between a TGN/endosomal loop and also between an early endosome/plasma membrane loop (for review, see Molloy et al., 1999). Transport in each cycling loop relies on binding of the PACS-1 adaptor to the phosphorylated form of furin and PACS-1, in turn, associates with the clathrin-associated sorting machinery (Wan et al., 1998; Crump et al., 2001). Dephosphorylation by protein phosphatase 2A of furin causes movement of furin from one loop to another (Molloy et al., 1998).

In yeast, phosphorylation has been shown to be important for routing of cell surface transporters and pheromone receptors into ubiquitin-dependent degradative vacuolar pathways (Hicke et al., 1998; Feng and Davis, 2000; Marchal et al., 1998). However, the role of phosphorylation in sorting of cargo that cycle between the Golgi and endosomes is unknown. In this study, we have investigated the role of potentially phosphorylatable residues in the cytosolic domain of Ste13p, both in the Ste13p and A-ALP contexts. We show for both proteins that mutation of a phosphorylation site prevents delivery into the PVC, indicating that phosphorylation regulates trafficking of A-ALP/Ste13p.

MATERIALS AND METHODS

General Methods and Antibodies

The production of minimal (synthetic dextrose) and rich (YPD) yeast media, the genetic manipulation of yeast strains, and all general molecular biology methods were performed as described previously (Ausebel et al., 2000) or as otherwise noted. Rabbit polyclonal antibodies against alkaline phosphatase (ALP) and Kex2p have been described previously (Nothwehr et al., 1996; Spelbrink and Nothwehr, 1999). Rabbit polyclonal antibodies against rabbit anti-Och1p were a gift from Vladimir Lupashin (University of Arkansas, Fayetteville, AR). Mouse antibodies against Vma2p, Vph1p, and Dpm1p were from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against alkaline phosphatase (ALP) and Kex2p have been described previously (Nothwehr et al., 1996; Spelbrink and Nothwehr, 1999). Rabbit polyclonal antibodies against rabbit anti-Och1p were a gift from Vladimir Lupashin (University of Arkansas, Fayetteville, AR). Mouse antibodies against Vma2p, Vph1p, and Dpm1p were from Molecular Probes (Eugene, OR) and rabbit anti-hemagglutinin epitope (HA) antibodies were from Covance (Richmond, CA).

Plasmids and Yeast Strains

Plasmids pSN34, pSN55, pSN100, pAH4, and pAH49 have been described previously (Nothwehr et al., 1993, 1999). pSN55-PS1 to pSN55-PS11 were made using site-directed mutagenesis (Kunkel et al., 1987) to introduce point mutations (detailed below) into pSN55, a pRS316 derivative containing a STE13-PHO8 gene fusion. A TRP1-based version of pSN55-PS2, called pSN397, was made by subcloning the 2.3-kbp Eagl-EcoRI fragment from pSN55-PS2 into the Eagl/EcoRI sites of pRS314. Plasmids pM36, pH73, and pH74, which are derivatives of pSN55 containing various combinations of Δ2-11, S1-A, S1-D, F2-A, and F2-A mutations, were made using the “megaprimer method” (Tyagi et al., 2004). Primer sequences are available upon request. The mutations were verified by DNA sequencing. Yeast strains used in this study are listed in Table 1.

Plasmid pSN286, used to introduce a GAL1-STE13 allele at the STE13 locus, consists of pRS306 (Sikorski and Hieter, 1989) containing an insert with the following elements in the order given: a 1.9-kbp BamHI-Eagl fragment from the 5′ untranslated region of STE13, a 1.1-kbp Eagl-BamHI fragment containing the GALI promoter, and a 2.7-kbp EcoRI-KpnI fragment containing the

Table 1. Yeast strains used in this study

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STEl3 open reading frame and 3’ untranslated region. Three derivatives of pSN286 contain the following point mutations in the STEl3 coding region: F335A (pH9262), and S337A (pH9263). (The four GAL1-STEl3 plasmids were introduced into yeast HY33 by first linearizing them at the unique SmaI site that lies in the 5’ untranslated region of STEl3; transforming them into yeast; selecting transformants on media lacking ura-
al; and finally, looping out intervening DNA on media containing 5-fluoro-
roic acid.

A GAL1 promoter-driven A-ALP construct tagged with two copies of the IgG-binding Z domain was generated by ligating the 2.25-kbp Eigh-Sall fragment (Nohwere et al., 2003) and a 1.2-kbp Sfl-Xbal fragment de-\nigested with (a gift from Per Stromhaug, University of Missouri, Columbia, MO) was digested with PacI, blunted as described above, digested with PacI, and the 0.7-kbp StuI fragment from pAH98 (Nothwehr et al., 2000) was cloned into the BamH1-XbaI site that lies in the 5’ coding region of the PHO8-GFP construct, was digested with XbaI to release the GFP region. Plasmid pFA6a-ZZ (a gift from Per Stromhaug, Professor of University of Missouri, Columbia, MO) was digested with PstI, blunted as described above, digested with PstI, and the 1.2-kbp SalI fragment was ligated to the pSN332 vector backbone fragment de-
scribed above, resulting in the GAL1-STEl3-PH08-ZZ construct pCP7.

Radioactive Labeling, Immunoprecipitation, and Western Blot Analysis

The procedure for immunoprecipitation of wild-type and mutant A-ALP from [35S]methionine/cysteine-labeled cells was performed as described pre-
viously (Nohwere et al., 1993). Radioactively labeled proteins were quantified-
fierically with the Phosphorimager system (Fuji Photo Film, Tokyo, Japan). For calculation of the half-time of A-ALP processing, the log of the percentage of A-ALP that was unprocessed at each time point was plotted as a function of time, and the plots were analyzed by linear regression analysis.

For detection of in vivo phosphorylation, cultures were grown for several doublings in phosphate-depleted rich (YPD) media (Warner, 1991) to log phase. Fifty to 100 μCi of [35P]Pi was then added to 0.5 OD600 units of cells, and the culture was incubated at 30°C for 45 min. The cells were then spheroplasted, pelleted, and subjected to immunoprecipitation according to (Shevchenko et al., 1996), and peptides were purified and de-
scribed previously (Nohwere et al., 1993). After separation by SDS-PAGE,uthesis in cell biology. Fluorescence Microscopy

The procedures for preparation of fixed spheroplasted yeast cells and attach-
ment to microscope slides were described previously (Roberts et al., 1991). All secondary antibodies were diluted 1:500 before use. Simultaneous detection of wild-type and mutant A-ALP and Vma2p was performed as described previously (Ha et al., 2003). Yeast cells were photographed using a BX-60 fluorescence microscope (Olympus, Lake Success, NY) equipped with a CFP42-95 digital camera (Hamamatsu, Bridgewater, NJ). Images were initially captured using Image 3.1.7 software (Improvision, Lexington, MA) and were processed into figures using Adobe Photoshop 7.0.

Mating Assay to Measure the Onset of Sterility

The mating efficiency of MATα yeast strains at various times after terminating expression of STEl3 and mutant derivatives under control of the GAL1 promoter was determined using a published assay (Hartwell, 1980; Wilcox et al., 1992). Briefly, cells were grown synthetic galactose media for several generations to log phase and were then switched to synthetic glucose (SD) media. After various times of growth in glucose, 0.25 OD600 units of cells (~5 x 107 cells) were mixed with 0.75 OD600 units of cells (~1.5 x 107 cells) of MATα mating type strain JHR20-Ca, and grown onto a 2.4-cm HAT filter (Millipore, Billerica, MA). The filters were incubated side up on YPD plates at 30°C for 4 h, the cells were resuspended off the filter, and the dilutions of cells were plated on SD-his media to select for MATa and diploid cells and onto SD-his-trp to select for diploids only. Mating efficiency is expressed as the number of diploids divided by the number of diploids plus MATα haploids.

Mass Spectrometry Analysis of Ste13p Cytosolic Domain Phosphorylation

The A-ALP protein fused to two copies of the IgG-binding Z domain was purified using yeast strain A-ALP carrying plasmid pCP7. In total, 100 OD600 units of cells was spheroplasted and lysed by incubating in 2.5 ml of 5% SDS/8 M urea at 100°C for 5 min in the presence of protease inhibitors (see above). The yeast extract was then diluted into 30 ml of immunoprecipitation (IP) buffer lacking SDS (10 mM Tris, pH 8.0, 0.1% Triton X-100, and 2 mM EDTA), and a 0.5-ml bed volume of Sepharose beads was added. After incubating for 1 h at 4°C, the beads were sedimented, and the supernatant was then added to 0.1-ml bed volume of IgG-Sepharose beads. After incuba-
tion, at 4°C, the beads were washed four times with volumes of IP buffer containing 0.1% SDS (Nohwere et al., 1993), incubated with SDS-PAGE sample buffer at 100°C, and the eluted protein separated by SDS-PAGE and stained with Coomassie Brilliant Blue. A gel band corre-
ding to full-length A-ALP-ZZ was subjected to in-gel trypsin digestion according to (Shevchenko et al., 1996), and peptides were purified and de-
scribed using Zip Tip columns (Millipore) according to the instructions of the manufacturer.

Subcellular Fractionation

Subcellular fractionation of [35S]-labeled cells by differential centrifugation was carried out as described previously (Nohwere et al., 1999), except that the immunoprecipitations were performed using anti-ALP antibody, and the pulse labelling (10 or 20 min) and the chase was for 0.5, 10, 20, and 40 min.

The nonradioactive subcellular fractionation experiment performed by dif-

ferential centrifugation was carried out as described previously (Ha et al., 2001), except centrifugation at 13,000 × g was used instead of 15,000 × g. Equi-

volumes of P13, P200, and S200 fractions were subjected to SDS-PAGE followed by blotting to nitrocellulose. The blots were probed with the indicated primary antibodies followed by incubation with ALP-conju-
gated anti-rabbit or anti-mouse secondary antibodies and were detected, quantified, and imaged as described above. Images were further adjusted and for PC7.

RESULTS

Yeast Resident TGN Proteins Are Phosphorylated

As a first step to investigate whether phosphorylation of TGN resident proteins is important for their trafficking, we assessed whether the model TGN protein A-ALP is phos-
phorylated. A-ALP is a model TGN-membrane protein consis-
ting of the N-terminal cytosolic domain of Ste13p fused to the transmembrane and lumenal domains of ALP, the PH08 gene product (Nohwere et al., 1993). A failure to retain A-ALP in the TGN/endosomal system results in its delivery to the vacuole where its C-terminal propeptide is proteolytically removed, resulting in a mobility shift on SDS-PAGE. However, to simplify the phosphorylation analysis, pep4 yeast strains deficient in vacuolar protease activity were used to prevent any vacuolar proteolytic processing from occurring. A yeast strain expressing A-ALP was grown in phosphate-depleted media for several generations and cells were then incubated with [35P]Pi, for 45 min followed by immunoprecipitation of A-ALP. A band of the expected size for A-ALP was obtained, whereas this band was missing in a strain expressing an A-ALP mutant lacking residues 2–100 of the cytosolic domain (Figure 1A, lanes 1 and 4). However, a very faint band at the size expected for A(Δ2-100)-ALP was...
observed. The extent of $^{32}$P incorporation into wild-type and mutant A-ALP was quantified and normalized to the amount of A-ALP protein immunoprecipitated (Figure 1B). These results indicated that phosphorylation of the A($\Delta 2$-100)-ALP mutant was reduced to $\sim$20% of that of A-ALP. We thus conclude that A-ALP is phosphorylated and that most, if not all, of the phosphorylation occurs on the cytosolic domain. In this regard it is worth noting that the truncated cytosolic domain in A($\Delta 2$-100)-ALP contains two Ser residues (Figure 2A). Kex2p (Figure 1A) and Vps10p (our unpublished data) also were demonstrated to be phosphoproteins.

We next addressed whether trafficking defects would affect phosphorylation of A-ALP. A mutant form of A-ALP, A(F$_{85}$A; F$_{87}$A)-ALP, defective for retrieval from the PVC (Nothwehr et al., 1993; Bryant and Stevens, 1997) was phosphorylated to a similar degree as A-ALP (Figure 1). Phosphorylation of A-ALP expressed in the class E vps mutant vps$^{4\Delta}$ also was analyzed. In class E mutants, transport into the PVC occurs normally but both retrograde and anterograde traffic out of the PVC is blocked (Piper et al., 1995; Babst et al., 1997; Finneneigen et al., 1997). Thus, in class E cells A-ALP rapidly becomes trapped in the PVC (Bryant and Stevens, 1997). However, loss of Vps4p function did not seem the extent of phosphorylation of A-ALP (Figure 1A).

**A S$_{13}$A Mutation in the Cytosolic Domain of A-ALP Blocks Its Delivery to the PVC**

The 24 potentially phosphorylatable Ser, Thr, and Tyr residues in the cytosolic domain of Ste13p/A-ALP were systematically mutated to determine whether they have a role in trafficking of A-ALP. In total, 11 mutant forms of A-ALP were generated by site-directed mutagenesis of one to four residues at a time (Figure 2A). These mutants, called PS1–PS11, and controls were expressed in a wild-type strain, cells were pulsed for 10 min, chased for 150 min, lysed, and then immunoprecipitated using anti-ALP antibody. Little or no vacuolar processing was observed, with the exception of PS3 that exhibited weak processing (Figure 2B). A defect in retrieval of A-ALP from the PVC results in processing with a half-time of $\sim$60 min (Nothwehr et al., 1993; Bryant and Stevens, 1997); thus, the mutations apparently do not have a marked effect on PVC-to-TGN retrieval.

To determine whether the mutations affect the rate of transport into the PVC, we expressed wild-type and mutant A-ALP proteins in the class E vps mutant vps$^{4\Delta}$ that trap TGN resident proteins in the PVC. In contrast to wild-type cells, the PVC in such strains contains substantial vacuolar protease activity. Therefore, the rate of processing of newly synthesized A-ALP in class E mutants reflects the rate of transport from its site of synthesis at the endoplasmic reticulum (ER) to the PVC (Bryant and Stevens, 1997; Ha et al., 2001). vps$^{4\Delta}$ cells carrying wild-type and mutant A-ALP proteins were pulsed for 10 min and chased for 60 min. Under these conditions, 71% of wild-type A-ALP was processed (Figure 2C). A mutant form of A-ALP lacking amino acids 2–11, A($\Delta 2$-11)-ALP, was more extensively processed (90%), consistent with previous work showing that this deletion accelerates trafficking into the PVC (Bryant and Stevens, 1997; Ha et al., 2001). Most of the PS mutants were processed to a similar degree as wild-type, suggesting that the mutated residues play little or no role in trafficking. Notable exceptions were the PS2 mutant (S$_{13}$A) that exhibited little or no processing, and the PS3 (S$_{13}$A, S$_{22}$A, S$_{24}$A, S$_{25}$A) and PS6 (T$_{27}$A, T$_{50}$A, T$_{64}$A) mutants whose processing was somewhat accelerated (82% processing). Because the

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Figure 1. The cytosolic domains of both A-ALP and Kex2p are phosphorylated in vivo. (A) Yeast strains were grown for several doublings in phosphate-depleted media and then continuously labeled with $[^{32}$P]$\Pi$ for 45 min. In lanes 1–7 strains LSY2/pSN55, LSY2/pSN55-PS2, LSY2/pSN100, LSY2/pSN34, PBY33/pSN55, LSY2, and AH48-13D were analyzed, respectively, by immunoprecipitation with anti-ALP antibodies (lanes 1–5) or with anti-Kex2p antibodies (lanes 6 and 7). (B) The strains analyzed in lanes 1–5 of A were labeled with $[^{32}$P] and subjected to immunoprecipitation of wild-type and mutant A-ALP proteins as described above. After separation by SDS-PAGE, the relative amounts of purified wild-type and mutant A-ALP proteins were quantified by immunoblotting, and the relative amounts of $^{32}$P incorporation were determined by PhosphorImager analysis as described under Materials and Methods. Relative phosphorylation is the extent of $^{32}$P incorporation divided by the level of wild-type or mutant A-ALP protein on the blot. Phosphorylation of wild-type A-ALP expressed in a wild-type strain was arbitrarily set at 100%. The average and SD of three independent data points for each strain are shown.

S$_{13}$A mutation caused such a striking effect on A-ALP trafficking, we compared A-ALP and A(S$_{13}$A)-ALP expressed in a vps$^{4\Delta}$ strain in a more extensive pulse-chase time course (Figure 2D). No processing of A(S$_{13}$A)-ALP was observed in the 80-min time course, whereas wild-type was processed with a half-time of 46 min (Table 2).
To determine whether the S13A mutation blocks delivery of A-ALP to the PVC in wild-type cells, we introduced this mutation into the A(F85A; F87A)-ALP construct that is incapable of being retrieved from the PVC. The rate of processing of A-ALP, A(F85A; F87A)-ALP, A(S13A)-ALP, and A(S13A; F85A; F87A)-ALP in wild-type cells was determined. Whereas retrieval-defective A(F85A; F87A)-ALP was processed with a half-time of 59 min, introduction of the S13A mutation into this protein dramatically delayed its processing with just a hint of processing at the 180-min time point (Figure 3 and Table 2). Consistent with the results of Figure 2B, no processing of the A(S13A)-ALP single mutant was observed.

**The S13 Residue of A-ALP Is Phosphorylated**

Given that A-ALP is phosphorylated and that the S13A mutation severely retards delivery to the PVC, we next attempted to assess whether the S13 residue is phosphorylated. If S13 is phosphorylated, then mutating it to a D residue to mimic the phosphorylated state would be expected to give a much different phenotype than the S13A mutant was indeed found to be processed significantly faster than A-ALP in **vps**4A cells (Figure 2D and Table 2). As another approach, we directly assessed the extent of in vivo phosphorylation of A(S13A)-ALP compared with wild-type. We have repeatedly observed a modest decrease in the extent of phosphorylation due to the S13A mutation although the extent of this decrease varies from experiment to experiment. In the experiment shown in Figure 1B, this difference fell within the SD due to quantitative limitations in the assay. If S13 is phosphorylated it is clearly not the only phosphorylation site in A-ALP, although the other unidentified site(s) do not seem to dramatically influence trafficking.

Given this uncertainty, mass spectrometry was used to address whether the S13 residue was phosphorylated. A-ALP fused to two copies of the IgG-binding Z domain was expressed in yeast, purified, and the protein contained within a one-dimensional gel band was trypsinized. Single-charge positive ions corresponding to the phosphorylated residue 12–19 peptide (at 1078.5 Da) and phosphorylated 11–19 or 12–20 peptides (at 1206.6 Da) were observed in the matrix-assisted laser desorption ionization/TOF MS analysis of both the nonbound and bound peptide fractions from a C18 matrix used for desalting the in-gel tryptic digest (our unpublished data). These ions were observed at significant signal-to-noise ratios only in the presence of the matrix 2,5-dihydroxybenzoic acid [at 10 mg/ml in 500:500:10 (vol/vol)] acetonitrile/water/o-phosphoric acid] and not with the matrix alpha-cyano-4-hydroxycinnamic acid. Subsequent nanospray quadrupole time of flight mass spectrometry analysis revealed a triple-charge ion at a mass/charge ratio (m/z) of 402.9 that corresponded well to the signal at m/z 370.2 and 358.9, which corresponded well to the expected m/z for a phosphorylated peptide corresponding to residues 11–19. The fragmentation spectrum of this peptide showed a complete y-ion series consistent with phosphorylation at S13 that included both phosphorylated fragment ions 11–19 and 12–20. Together, these results strongly suggest that phosphorylation at S13 controls delivery of A-ALP/Ste13p to the PVC.
The results mentioned above suggested that the S13 residue is required for trafficking of A-ALP into the PVC. To explore the alternative possibility that the S13A mutation converts A-ALP into an unsuitable substrate for vacuolar processing, we assessed the localization of A(S13A)-ALP in wild-type and vps4Δ cells by immunofluorescence microscopy (Figure 5). In wild-type cells, both A-ALP and A(S13A)-ALP exhibited punctate staining patterns typical of localization to the yeast Golgi/endosomal system. However, extensive analysis of many fields of cells indicated that the staining pattern of A(S13A)-ALP was subtly different in that it decorated structures that seemed slightly smaller and more numerous than A-ALP (unpublished data). The exaggerated PVC processing compartment in vps4Δ cells is marked by Vma2p, a component of the vacuolar ATPase (Raymond et al., 1992). Although wild-type A-ALP clearly colocalizes with Vma2p in vps4Δ cells, indicating that it is trapped in the exaggerated PVC, A(S13A)-ALP remains punctate in the vps4Δ cells. Thus, the lack of processing of A(S13A)-ALP in vps4Δ cells is due to its lack of transport to the PVC.

The punctate staining pattern suggested that A(S13A)-ALP had reached the TGN/endosomal system; however, an alternative possibility was that this mutant protein was trapped in the ER. To explore this issue, we used a differential centrifugation approach to fractionate organelles from lysates derived from wild-type cells expressing A-ALP and cells expressing A(S13A)-ALP. Lysates were centrifuged at 13,000 × g to generate a pellet (P13) and supernatant (S13) fraction. The S13 fraction was then centrifuged at 200,000 × g to generate P200 and S200 fractions. Under these conditions A-ALP, A(S13A)-ALP, and Kex2p were mainly found in the P200 fraction with a minor amount in the P13 fraction (Figure 6). In contrast, the ER marker Dpm1p and vacuolar marker Vph1p were clearly enriched in the P13 fraction. These results indicated that A(S13A)-ALP was not localized to the ER in the steady state and were consistent with a Golgi/endosomal localization.

The S13A Mutation Does Not Slow the Rate of ER-to-Golgi Transport of A-ALP

Although A(S13A)-ALP was not localized to the ER under steady-state conditions, it was possible that newly synthesized A(S13A)-ALP transits slowly through the early part of the secretory pathway. If so, this could partially account for the slow rate of transport into the PVC. To investigate this issue, we performed subcellular fractionation on strains expressing A-ALP and A(S13A)-ALP that had been pulse labeled for 10 min and chased for 0, 20, and 40 min (Figure 7). By the earliest time point measured (0 min), the majority of A-ALP and A(S13A)-ALP were already found in the P150 fraction with only minor amounts in the P13 fraction. As in Figure 6, the ER marker Dpm1p in this experiment was confined almost completely to the P13 fraction, with little or no Dpm1p in the P150 and S150 fractions (Figure 7B). Proceeding through the 0- to 40-min time course, little or no additional shift of A-ALP and A(S13A)-ALP from the P13 to the P150 fraction was observed. Although this type of experiment is not capable of ruling out very minor differences in ER-to-Golgi trafficking rates, it is clear that both proteins were transported from the ER to the Golgi in <10 min after being synthesized. Together, these results indicate that the block into transport of A(S13A)-ALP into the PVC must be due to an effect on intra-Golgi or post-Golgi trafficking.

The S13A Mutation Suppresses the Accelerated Depletion of PVC Retrieval-Defective Ste13p from the α-Factor Processing Compartment

The S13A mutation could cause A-ALP to limit its cycling to a TGN/early endosome itinerary. Alternatively, A(S13A)-ALP could be localized to a post-TGN non-PVC endosomal organelle distinct from the α-factor processing compartment. To investigate this issue, and to extend our analysis to endogenous Ste13p, we used a functional assay that measures the persistence of wild-type and mutant forms of Ste13p in the α-factor processing compartment (the TGN and/or early endosome). We hypothesized that retrieval-defective Ste13p (F85A; F87A) would be depleted from the TGN/early endosome more rapidly after its synthesis was shut-off than wild-type Ste13p but that the S13A mutation would block entry into the PVC and thus suppress this

Table 2. Rate of processing of wild-type and mutant A-ALP proteins in various strain backgrounds

<table>
<thead>
<tr>
<th>Strain</th>
<th>Form of A-ALP</th>
<th>Half-time of processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Wild type</td>
<td>F85A; F87A</td>
<td>59 ± 4 n = 2</td>
</tr>
<tr>
<td>Wild type</td>
<td>S13A</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Wild type</td>
<td>S13A; F85A; F87A</td>
<td>&gt;80 n = 2</td>
</tr>
<tr>
<td>imp53Δ</td>
<td>F85A; F87A</td>
<td>48</td>
</tr>
<tr>
<td>imp53Δ</td>
<td>S13A</td>
<td>35</td>
</tr>
<tr>
<td>imp53Δ</td>
<td>S13A; F85A; F87A</td>
<td>35 n = 2</td>
</tr>
<tr>
<td>vps1Δ</td>
<td>wild type</td>
<td>62 ± 3 n = 2</td>
</tr>
<tr>
<td>vps1Δ</td>
<td>S13A</td>
<td>&gt;80</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>Wild type</td>
<td>46 ± 4 n = 5</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>S13A</td>
<td>&gt;80</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>S13D</td>
<td>32 ± 1 n = 2</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>Δ2-11</td>
<td>22 ± 8 n = 2</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>Δ2-11; S13A</td>
<td>23 ± 6 n = 2</td>
</tr>
<tr>
<td>vps4Δ</td>
<td>Δ2-11; S13D</td>
<td>19 ± 3 n = 2</td>
</tr>
</tbody>
</table>

Strains of the indicated genotype (SHY35, SHY38, SHY39, and SNY148) carrying plasmids for expression of wild-type (pSN55), F85A; F87A (pSN100), S13A (pSN55-PS2), S13A; F85A; F87A (pHJ63), S13A; F85A; F87A (pHJ75), Δ2-11 (pAH49), Δ2-11; S13A (pHJ73), and Δ2-11; S13D (pHJ74) forms of A-ALP were analyzed. Strains were pulsed with [35S]methionine/cysteine for 10 min and chased for a total of four time points at 30°C. After each time point, wild-type or mutant A-ALP proteins were immunoprecipitated, separated by SDS-PAGE, the percentage of processing quantified by Phosphorimager analysis, and the processing half-time calculated (see Materials and Methods). For data points derived from multiple experiments (n > 1), the average and SD are indicated.

Figure 3. The S13A mutation blocks delivery of A-ALP to the PVC in wild-type cells. Strain SHY35 carrying pSN55 (A-ALP), pSN100 [A(F85A; F87A)-ALP], pSN55-PS2 (A(S13A)-ALP), and pHJ63 [A(S13A; F85A; F87A)-ALP] were pulsed for 10 min and chased for 0, 45, 90, and 180 min. Little or no processing of the double mutant is seen, even after 180 min, suggesting that the S13A mutation blocks delivery into the PVC in wild-type cells.

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The A(S13A)-Alkaline Phosphatase Mutant Exhibits a TGN/Endosomal-like Distribution

The results mentioned above suggested that the S13 residue is required for trafficking of A-ALP into the PVC. To explore the alternative possibility that the S13A mutation converts A-ALP into an unsuitable substrate for vacuolar processing, we assessed the localization of A(S13A)-ALP in wild-type and vps4Δ cells by immunofluorescence microscopy (Figure 5). In wild-type cells, both A-ALP and A(S13A)-ALP exhibited punctate staining patterns typical of localization to the yeast Golgi/endosomal system. However, extensive analysis of many fields of cells indicated that the staining pattern of A(S13A)-ALP was subtly different in that it decorated structures that seemed slightly smaller and more numerous than A-ALP (unpublished data). The exaggerated PVC (class E compartment) in vps4Δ cells is marked by Vma2p, a component of the vacuolar ATPase (Raymond et al., 1992). Although wild-type A-ALP clearly colocalizes with Vma2p in vps4Δ cells, indicating that it is trapped in the exaggerated PVC, A(S13A)-ALP remains punctate in the vps4Δ cells. Thus, the lack of processing of A(S13A)-ALP in vps4Δ cells is due to its lack of transport to the PVC.

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effect. Strains were constructed in which the endogenous STE13 gene was replaced with constructs containing the galactose-inducible GAL1 promoter fused to the following alleles of the STE13 gene: 1) wild-type, 2) F85A; F87A, 3) S13A, and 4) S13A; F85A; F87A. These strains were grown on galactose media to induce expression of wild-type and mutant Ste13p, switched to glucose media for various times, and then were tested for their ability to mate with a MATa strain (Figure 8). The efficiency of mating of the F85A; F87A mutant did indeed drop off more quickly than wild type after the shift to glucose, as can be seen at the 6- and 12-h time points. The Ste13 (S13A) single mutant persisted in the TGN/early endosome to a similar degree as wild type over the time course. In addition, the S13A muta-

Figure 4. The S13 residue in the Ste13p cytosolic domain is phosphorylated. The MS/MS fragmentation spectra and amino acid sequence of a trypsin-miscleaved peptide from the Ste13p cytosolic domain beginning with Lys11 is shown. The intact 3+ charged parent ion (y3) had a mass/charge ratio (m/z) of 402.86. Peaks corresponding to the y (C-terminal) and b (N-terminal) fragment ions (inset) are labeled on the plot. An exception is the relatively small peak for the y5 ion with an m/z of 399.22 that could only be discerned on an expanded version of the plot. The ion fragments with an expected mass resulting from neutral loss of H3PO4 are marked with asterisks. The y3 fragment was identified in two distinct peaks with one corresponding to a 1+ charged ion (m/z = 400.24) and the other to a 2+ charged ion (m/z = 200.62) species.
A(S13A)-ALP fractionates similarly to wild-type A-ALP by differential centrifugation of cell lysates. Strains SHY35/pSN55 and SHY35/pSN55-PS2 were spheroplasted, lysed, and lysates were subjected to centrifugation at 13,000 × g to generate pellet (P13) and supernatant (S13) fractions. The S13 fraction was then centrifuged at 150,000 × g to generate P150 and S150 fractions. Equivalent percentages of the three fractions from each strain were run on SDS-PAGE, gels were transferred to nitrocellulose, and blots were probed using antibodies to detect the indicated proteins.

The S13 Phosphorylation Site Is Necessary for Plasma Membrane-mislocalized A-ALP to Reach the PVC

Loss of function of clathrin heavy chain, Chc1p, and the dynamin homolog Vps1p block direct Golgi-to-endosome trafficking of A-ALP. In chc1-521ts and vps1Δ mutants, A-ALP is initially mislocalized to the plasma membrane and is then delivered to the vacuole in a manner dependent on the End3 and Sla2/End4 proteins (Nothwehr et al., 1995; Ha et al., 2003). End3p and Sla2p are required for the internalization step of endocytosis (Raths et al., 1993; Bénédetti et al., 1994). Presumably in vps1Δ and chc1Δ mutants A-ALP vacuolar delivery from the plasma membrane occurs via the early endosome and endosome-to-TGN retrieval is compromised in these mutants. We assessed whether A(S13A)-ALP could access the PVC/vacuole after it was mislocalized to the plasma membrane in chc1-521ts and vps1Δ mutants. Wild-type A-ALP was clearly processed in vps1Δ cells and in chc1-521tsΔ mutants at the nonpermissive temperature within the 80-min time course (Figure 9, A and B) as has been observed previously (Nothwehr et al., 1995; Ha et al., 2003). However, processing of A(S13A)-ALP was blocked in these mutants.

A pool of A-ALP is trapped on the plasma membrane in vps1Δ cells with a loss of End3p or Sla2p function (Nothwehr et al., 1995, 1999). If A(S13A)-ALP also is mislocalized to the plasma membrane in vps1Δ cells, it should be trapped there in vps1Δ end3-ts cells. To investigate this, we pulse labeled A-ALP and A(S13A)-ALP as a percentage of the total found in the P13, P150, and S150 fractions at each time point. The amount of A-ALP or A(S13A)-ALP found in the S150 was <5% of the total found in the P13, P150, and S150 fractions combined. (B) Equivalent percentages of the 0 min P13, P150, and S150 fractions (nonimmunoprecipitated) were run on SDS-PAGE and were Western-blotted for Dpm1p. Fractions from strain SHY35/pSN55 and strain SHY35/pSN55-PS2 are shown in lanes 1–3 and 4–6, respectively.

Figure 7. The S13A mutation does not alter the kinetics of A-ALP trafficking from the ER to the Golgi. (A) Strains SHY35/pSN55 (A-ALP) and SHY35/pSN55-PS2 [A(S13A)-ALP] were spheroplasted and pulse labeled for 10 min and were chased for 0, 20, and 40 min. After each chase period, the cells were lysed and lysates were centrifuged at 13,000 × g to generate pellet (P13) and supernatant (S13) fractions. The S13 fraction was then centrifuged at 150,000 × g to generate P150 and S150 fractions. A-ALP or A(S13A)-ALP were immunoprecipitated from each fraction, analyzed by SDS-PAGE, and quantified by PhosphorImager analysis. The numbers below each panel represent the amount of the A-ALP or A(S13A)-ALP present in the P13 and P150 fractions as a percentage of the sum total present in the P13 and P150 fractions. At each time point, the amount of A-ALP or A(S13A)-ALP found in the S150 was <5% of the total found in the P13, P150, and S150 fractions combined. (B) Equivalent percentages of the 0 min P13, P150, and S150 fractions (nonimmunoprecipitated) were run on SDS-PAGE and were Western-blotted for Dpm1p. Fractions from strain SHY35/pSN55 and strain SHY35/pSN55-PS2 are shown in lanes 1–3 and 4–6, respectively.

Loss of function of the synaptojanin homolog Inp53/Sjl3p causes A-ALP transport into the PVC to be accelerated (Ha et al., 2001, 2003). To investigate the epistatic relationship between INP53 and the S13a phosphoserine, we analyzed the rate of processing of retrieval defective A(F85A; F87A)-ALP in inp53Δ cells with or without the S13a mutation. As observed previously, A(F85A; F87A)-ALP was processed faster in inp53Δ cells (35 min) than in wild-type cells (59 min; Table 2). However, processing of the F(S13A; F85A; F87A)-ALP mutant was blocked in inp53Δ cells as well as in wild-type cells. Interestingly, the acceleration of trafficking into the PVC caused by the S13Δ mutation was additive to that caused by the inp53Δ mutation with the processing half-time...
for the S13D; F85A; F87A triple mutant in wild-type cells (48 min) decreasing in inp53Δ cells (28 min). These data indicate that Inp53p mediates trafficking of A-ALP in a manner dependent on the phosphoserine.

The Block in A-ALP Trafficking Caused by the S13A Mutation Requires the 2–11 Region

Deletion of residues 2–11 of A-ALP accelerates its transport into the PVC (Bryant and Stevens, 1997; Ha et al., 2001); thus, this region may contain a signal to slow transport into the PVC. The S13D mutation constructed to mimic hyperphosphorylation accelerates A-ALP trafficking to the PVC, whereas the S13A mutation that mimics unphosphorylated serine blocks this trafficking step. Given the contrast in phenotype between the Δ2-11 and S13A mutants, we tested the possibility that the phosphoserine and 2–11 motifs antagonize each other.

The rate of processing of single and double mutant A-ALP constructs was assessed in the vps4Δ mutant that provides a measure of the rate of trafficking into the PVC. Interestingly, in vps4Δ cells the S13A; Δ2-11 double mutant was processed more rapidly than wild-type, with kinetics similar to the Δ2-11 single mutant (Table 2). Similar results were obtained for the Δ2-11; S13D double mutant. Therefore, these double mutant experiments indicate that the Δ2-11 mutation neutralizes the effect that mutating S13 has on the rate of trafficking into the PVC, consistent with the idea that the phosphoserine may act by antagonizing the 2-11 signal.

DISCUSSION

This study demonstrates for the first time that phosphorylation influences trafficking of a yeast resident TGN membrane protein. Mutation of S13 to A in the cytosolic domain of A-ALP did not slow trafficking to the Golgi. However, this mutation dramatically inhibited the normal delivery of A-ALP to the PVC under several different conditions. The notion that the S13A mutation simply converts A-ALP to an unsuitable proteolytic substrate can be ruled out because the A(S13A)-ALP was clearly shown to exhibit a non-PVC, Golgi-like localization pattern in class E vps4 cells, whereas wild-type A-ALP was predominantly associated with the PVC in vps4 mutants. In addition, we demonstrate that the phosphorylation state of S13 regulates A-ALP trafficking. First, analysis of peptides containing S13 by mass spectrometry demonstrated that this serine residue is indeed phosphorylated. Second, mutation of S13 to D to mimic phosphorylation accelerated trafficking into the PVC, whereas the S13A mutation to mimic unphosphorylated serine retarded trafficking into the PVC.

New insights into how yeast TGN membrane proteins reach the PVC have been gained in recent years, and these are relevant to understanding the role of S13 phosphorylation. A variety of evidence suggests that TGN membrane proteins reach the PVC via an early endosomal compartment (see Introduction). Whether A-ALP and Kex2p also use the GGA-dependent direct TGN-to-PVC pathway is less clear. A loss of function of the GGA proteins clearly delays vacuolar processing of Cps1p (Costaguta et al., 2001) consistent with the idea that Cps1p relies on the direct TGN-to-PVC pathway. However, the loss of GGA protein function does not affect the rate of trafficking of A(F85A; F87A)-ALP trafficking into the PVC/vacuole (Ha et al., 2003). Trafficking of A(F85A; F87A)-ALP into the PVC/vacuole in gga1Δ mutants does not occur via the plasma membrane because it is not blocked by an end3-ts mutation (Foote and Nothwehr, unpublished data). Together, these results suggest either

Figure 8. The S13A mutation suppresses the accelerated loss of retention in the α-factor processing compartment observed for Ste13p carrying the F85A; F87A mutations. Strains HJY4, HJY5, HJY6, and HJY7 carrying wild-type and mutant forms of the STE13 under control of the GAL1 promoter were grown on galactose for several generations. The strains were then shifted to glucose-containing media to turn off expression of the wild-type and mutant STE13 constructs. At the indicated time points after shifting to glucose the ability of the cells to mate was measured to generate an index of mating efficiency (for details, see Materials and Methods). The data shown are the average of three independent data points and the error bars indicate the SD.
that A-ALP and Kex2p do not normally use the GGA-dependent direct TGN-to-PVC pathway or they do use this pathway to a limited degree but alternatively use the TGN-to-early endosome pathway if the GGA pathway is blocked. A definitive resolution to this issue will require thorough analysis of the transport vesicles from these pathways and their contents.

To gain clues about the function of the S13 phosphoserine, we attempted to determine where the A(S13A)-ALP protein was localized. A(S13A)-ALP exhibited a punctate staining appearance typical of Golgi/endosomal proteins. However, the structures containing A(S13A)-ALP seemed slightly smaller and more numerous than those containing A-ALP. Nevertheless, equilibrium density gradient analysis demonstrated that A(S13A)-ALP associated with membranes having a density indistinguishable from that of A-ALP (Johnston and Nothwehr, unpublished data). Thus, the basis for the slightly different staining pattern of A-ALP and A(S13A)-ALP is presently unclear. In this regard, the S13A mutation does not preclude localization to the compartment(s) where α-factor maturation occurs because Ste13p carrying the S13A mutation exhibited α-factor processing efficiency that was similar to wild-type Ste13p. Furthermore, the rates of depletions of wild-type and S13A mutant Ste13p out of the processing compartment after their synthesis was stopped were very similar. Consistent with the A-ALP processing results, the S13A mutation in Ste13p suppressed the effect of the F85A; F87A mutations, indicating that the S13A mutation inhibited delivery of Ste13p into the PVC. Together, the results imply for A-ALP/Ste13p that both the wild-type and S13A mutants populate the TGN/early endosome. Rather, the difference between the wild-type and mutant proteins seems to involve the wild-type protein frequently cycling via the PVC, whereas the S13A mutant does not.

A loss in Inp53p function causes accelerated transport of A-ALP into the PVC (Ha et al., 2001). Epistasis analysis between the S13A mutations and the inps3Δ mutation was carried out to gain clues about the relative order of function of the phosphoserine and Inp53p. Like wild-type cells, inps3Δ cells exhibited a block in delivery of A(S13A)-ALP to the PVC. Interestingly, a small additive effect was observed between S1ΔD and inps3Δ. These data suggest that the acceleration of A-ALP through the pathway that occurs due to loss of Inp53p function occurs before (or at) the step in which the phosphoserine is required. A previous study showed that processing of retrieval-defective A-ALP was severely retarded in an inps3Δ gga1Δ gga2Δ triple mutant (Ha et al., 2003). A model proposed in that study was that A-ALP reached the PVC more rapidly in inps3Δ single mutants than the triple mutant because it used the direct TGN-to-PVC pathway by default. The implication was that Inp53p was needed to restrict trafficking to the TGN-to-early endosome pathway. However, the present study would seem to rule out such a model. The S13A mutation seems to affect trafficking at the early endosome (see below). Therefore, if A-ALP were using the direct TGN-to-PVC pathway in inps3Δ mutants then the S13A mutation would not be expected to have an effect on A-ALP trafficking in inps3Δ cells. The data suggest instead that loss of Inp53p function could reduce static retention in the TGN/early endosome so that A-ALP rapidly enters the PVC in a phosphoserine-dependent manner. The reason for the delay in retrieval defective A-ALP processing in the inps3Δ gga1Δ gga2Δ triple mutant is not known but these mutations also caused a severe growth defect and the slow
trafficking could be due to more broad-ranged defects in the TGN/endosomal system.

To learn more about the trafficking step affected by the S$_{13}$$\Delta$ mutation, we tested whether trafficking of A(S$_{13}$$\Delta$)-ALP from the plasma membrane to the PVC could occur. This was accomplished using the vps1$\Delta$ and chc1-521$^{*}$ mutations that mislocalize A-ALP to the plasma membrane whereupon A-ALP is then delivered to the vacuole, presumably via the PVC. The A(S$_{13}$$\Delta$)-ALP was shown to mislocalize to the plasma membrane, but unlike A-ALP, it was not subsequently delivered to the vacuole. Together with the other results of this study, the most straightforward interpretation of this result is that the early endosome-to-PVC transport step is abolished for A(S$_{13}$$\Delta$)-ALP. These results imply that the phosphoserine is needed at the level of an early endosomal compartment for subsequent delivery to the PVC.

Two sorting signals have previously been characterized in the A-ALP cytosolic domain. The FXFXD motif associates with the retromer to mediate PVC-to-TGN retrieval (Nothwehr et al., 2000), whereas a poorly characterized signal in the 2–11 region somehow regulates entry into the PVC. The 2–11 signal was identified based on the observation that deletion of this region accelerated A-ALP transport into the PVC (Bryant and Stevens, 1997). A similar region in Kex2p (TLS2) has been proposed to function by regulating the partitioning of Kex2p between the TGN-to-early endosome (EE) pathway and the direct TGN-to-PVC pathway (Sipos et al., 2004). According to this model, mutation of TLS2 causes Kex2p to predominantly use the direct TGN-to-PVC pathway. Recent data seem to argue against this type of role for the 2–11 signal of A-ALP. Analysis of trafficking of A-ALP and A($\Delta$$\Delta$-11)-ALP into the PVC in a gga1,$\Delta$ end3-ts strain, which should limit trafficking to the TGN/early endosome/PVC route, revealed that deletion of 2–11 accelerated trafficking into the PVC (Nothwehr, unpublished data). Thus, it is plausible that the 2–11 signal could function in static retention at the TGN/early endosome or in retrieval from the early endosome back to the TGN (but see below).

Although the 2–11 region is necessary to slow delivery into the PVC, it is not known whether this region is sufficient for this function or whether other motifs may regulate its function. Thus, it is possible that a true signal slowing transport into the PVC might lie outside of this region. One promising possibility is that the 2–11 region somehow regulates entry into the PVC. The A(S$_{13}$$\Delta$)-ALP was shown to mislocalize to the plasma membrane, and A-ALP is then delivered to the vacuole, presumably via the PVC. The A(S$_{13}$$\Delta$)-ALP was shown to mislocalize to the plasma membrane, but unlike A-ALP, it was not subsequently delivered to the vacuole. Together with the other results of this study, the most straightforward interpretation of this result is that the early endosome-to-PVC transport step is abolished for A(S$_{13}$$\Delta$)-ALP. These results imply that the phosphoserine is needed at the level of an early endosomal compartment for subsequent delivery to the PVC.

Several animal cell cargo proteins contain phosphorylation sites embedded with an acidic cluster motif that, when phosphorylated, enhance binding of adaptors and other sorting machinery. In addition to the furin example (see Introduction), phosphorylation of the mannose 6-phosphate receptor increases affinity for the GGA proteins by approximately threefold (Kato et al., 2002). Similarly, AP-1 and AP-2 binding to the T-cell coreceptor CD4 via a dileucine motif is dramatically enhanced by phosphorylation at nearby Ser residues (Pitcher et al., 1999). However, models discussed here for the S$_{13}$ residue of Ste13p/A-ALP instead posit that phosphorylation may decrease affinity for a sorting factor. In this regard it is worth noting that the 2–11 region is highly basic. Thus, the efficiency of interaction between a sorting factor and the basic 2–11 region might rely on electrostatic interactions could be reduced by the presence of a negatively charged phosphoserine at position 13.

What purpose might be served by using phosphorylation as way of regulating trafficking of a TGN membrane protein? An interesting possibility is that this may allow the cell to alter trafficking patterns of certain proteins as a function of environmental or intrinsic conditions e.g., stress, nutrient availability, pheromone response, cell cycle etc. A few examples of this type of regulation are known in yeast. For example, cell surface receptors such the uracil permease and a-factor receptor also are phosphorylated which positively regulates their ubiquitin-mediated internalization (Hicke et al., 1998; Marchal et al., 2000, 2002). The localization of the yeast glycosyltransferase Mnn1p to early Golgi compartments is controlled by the high osmolarity glycerol mitogen-
activated protein kinase pathway (Reynolds et al., 1998), presumably to integrate osmotic stress responses with localization of enzymes such as Mnn1p that are important for cell wall biosynthesis. Moreover, osmotic stress also causes yeast chitin synthase Chs3p to be transported from an early endosomal compartment to the plasma membrane, an event regulated by a protein kinase C pathway (Valdivia and Schekman, 2003). Thus, it is possible that the pheromone processing enzymes Ste31p and Kex2p also might be regulated by cell signaling pathways that could affect the production of mature pheromone or other proteins that are dependent on processing by these enzymes. Testing of this idea awaits identification of the kinase and phosphatase for the $S_{33}$ residue of Ste13p/A-ALP and characterization of the upstream regulatory machinery.

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